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Detection of *Clostridium botulinum* Toxin A Using a Fiber Optic-Based Biosensor

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A rapid, sensitive, analytical method for the detection of *Clostridium botulinum* toxin has been developed. The fiber optic-based biosensor utilizes the evanescent wave of a tapered optical fiber for signal discrimination. A 50 mW argon-ion laser, which generates laser light at 514 nm, is used in conjunction with an optical fiber probe that is tapered at the distal end. Antibodies specific for *C. botulinum* are covalently attached to the surface of the tapered fiber. The principle of the system is a sandwich immunoassay using rhodamine-labeled polyclonal anti-toxin A immunoglobin G (IgG) antibodies for generation of the specific fluorescent signal. Various anti-toxin antibodies were immobilized to the fibers. Affinity-purified polyclonal horse anti-toxin A antibodies performed better than the IgG fraction from the same horse serum or than the monoclonal anti-toxin A antibody BA11-3. Botulinum toxin could be detected within a minute, at concentrations as low as 5 ng/ml. The reaction was highly specific and no response was observed against tetanus toxin.

Fiber optic-based biosensors utilizing the evanescent wave of a fiber optic waveguide are being developed for the detection of serum analytes, environmental pollutants, and a host of other compounds (1,2). Fiber optic fluorescent evanescent wave sensors recently developed by Walczak *et al.*, and Rogers *et al.*, have shown sensitivity levels in the picomolar range for the detection of the MB isoenzyme of creatine kinase and the nanomolar range for α-bungarotoxin, respectively (3,4).

A fluorescence-based fiber optic fluorimeter has recently been developed (5) and used with immunoassays. The system utilizes the evanescent wave of a tapered fiber optic waveguide (6) in combination with a sandwich immunoassay performed at the surface of the tapered optical fiber. The evanescent wave allows the sensor to monitor antigen–antibody reactions in real time without washing or separation steps. Quantitative results are available within minutes of sample introduction. To simplify the handling of hazardous samples, fiber lengths can be varied so that testing can be performed in a location separate from the optics. This biosensor was adapted for the detection of *Clostridium botulinum* neurotoxin.

Neurotoxins produced by *C. botulinum* act at the level of the neuromuscular junction and upon ingestion by humans can be fatal. The mouse bioassay (7,8) is currently the most sensitive and widely accepted method for detecting botulinum toxin in serum and food extracts. This method takes 4 days to perform, requires a large number of mice if the toxin is to be quantified, and can only be made specific by toxin neutralization tests performed in conjunction with the assay. Enzyme-linked immunosorbent assays have been reported that are capable of detecting around 100 mouse lethal doses per milliliter (MLD_{50} units/ml)[1] (9,10). Recent improvements in these assays by enzyme amplification schemes have increased sensitivity to a range of 10–20 MLD_{50} units/ml (11–13). However, these types of assays still require about 6 h to complete and are not widely accepted as being sensitive enough to replace the mouse bioassay.

In the studies described here, the sandwich immunoassay was performed as a one-step or two-step immunoassay with similar results. In the one-step assay, the fluorescent antibodies, toxin, and the fiber optic probe are incubated together. In the two-step procedure, the fiber optic probe is first incubated with the toxin. The

[1] Abbreviations used: MLD, mouse lethal dose; IgG, immunoglobin G; MAb, monoclonal antibody; PBS, phosphate-buffered Saline; BBS, borate-buffered saline; TRITC, tetramethylrhodamine-5-isothiocyanate; BSA, bovine serum albumin; S/N, signal to noise.

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fiber is removed and placed in the solution of fluorescent antibodies. Various anti-toxin A antibodies were immobilized on the optical fibers, and assays using different concentrations of toxin A were performed. Immobilized polyclonal, affinity-purified or monoclonal anti-toxin A IgG antibodies were compared for their ability to act as capture reagents for holding a fluorophore-labeled antibody–toxin complex within the evanescent wave region of an optical fiber.

MATERIALS AND METHODS

Safety Procedures

Personnel working with botulinum toxin were immunized with pentavalent botulinum toxoid (IND 161, CDC) and procedures recommended in Biosafety in Microbiological and Biomedical Laboratories (HHS Publication (NIH) 88-8395, 1988) were followed.

Purification of Polyclonal and Monoclonal Antibodies

Monoclonal antibody, BA11-3 (MAb BA11-3) (14), and serum from horses hyperimmunized with *C. botulinum*, type A or B, were obtained from USAMRIID, Fort Detrick (Frederick, MD). All antibodies utilized in this study were purified with protein G–Sepharose purchased from Genex Corp. (Gaithersburg, MD). Briefly, columns were equilibrated with 0.01 M phosphate-buffered saline (PBS), pH 7.0. Horse serum or ascites fluid, diluted 1:2, was applied directly to the columns and washed with PBS. Antibodies were eluted from the column with 0.5 M ammonium acetate, pH 3.0, and pooled fractions dialyzed against PBS.

Affinity Purification of Anti-botulinum Toxin A Antibody

To prepare the affinity column, tresyl chloride-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) was washed in 1 N HCl and rinsed with coupling buffer (0.1 M sodium bicarbonate, pH 9.0, containing 0.5 M NaCl). *C. botulinum* toxin, type A, was purified following the procedure of Dasgupta and Sathyamoorthy (15).

Toxin A was added to 5 ml of coupling buffer and mixed at a ratio of 1 mg toxin per 0.5 g Sepharose 4B. The mixture was rocked overnight at 4°C. The Sepharose was washed in coupling buffer and any remaining reactive groups were blocked with 0.1 M Tris–HCl buffer, pH 8.0.

For antibody purification, 2.0 mg of horse IgG was incubated with the botulinum type A–Sepharose for 2 h. The column was washed with 0.15 M PBS, pH 7.4. Antibodies were eluted from the affinity column using 0.1 M glycine, pH 2.0, and dialyzed in PBS.

Fluorophore Labeling of Horse IgG Antibodies

Anti-toxin A IgG was dialyzed against 0.05 M borate-buffered saline (BBS), pH 9.3, containing 0.04 M NaCl. Tetramethylrhodamine-5-isothiocyanate, isomer G (Molecular Probes, Eugene, OR) (TRITC), was dissolved in dimethylformamide (3 mg in 0.2 ml) and added to 100 ml of BBS. The dialysis bag containing approximately 2 mg of IgG was placed in the TRITC solution and stirred at 4°C for 12 h. The TRITC-conjugated IgG was separated from free TRITC using a Sephadex G-25 column (Pharmacia, Piscataway, NJ). Protein concentrations and TRITC/IgG ratios were calculated based upon absorption values at 280, 515, and 555 nm as described by Amante *et al.* (16). The ratio of fluorophore to protein was 2:1.

Fiber Preparation

Fused silica fibers (plastic clad), 200 μm in core diameter, were obtained from Quartz et Silice (Quartz Products, Tuckerton, DE). Fibers were cut in 1.0-m lengths. At one end a SMA-metal connector (905-150-5003) from Amphenol Fiber Products (Lisle, IL) was attached using a crimped metal sleeve and heat shrink tubing. A small amount of epoxy was used inside the connector for additional reinforcement. The fiber was polished at the connector end with a Buehler Fibremet polishing device. The polished surface of the connector end was roughened by placement in a 3:1 mixture of concentrated HCl and nitric acid for 20 min (17). The metal surface was painted with flat black enamel paint.

Tapered fibers have been shown to increase the sensitivity of this device by a factor of 10 (6). Therefore, cladding from the distal 12 cm of the fiber was stripped away using a razor blade and the exposed core was continuously tapered by slow immersion into hydrofluoric acid and cleaved to 10 cm. The tapered end was then acid cleaned and treated for the covalent attachment of antibodies as described by Bhatia *et al.* (18). A thiol-terminal silane layer and a heterobifunctional cross linker, *N*-γ-maleimidobutyryloxy succinimide ester, were used in this covalent attachment procedure. Horse polyclonal IgG, affinity-purified anti-toxin A antibody, or MAb BA11-3 were immobilized on the fibers using 0.05 mg/ml solutions. This procedure has been used reproducibly to immobilize proteins onto fused silica fibers at a final density of 2 ng/mm^2 IgG protein with a binding capacity for large molecular weight antigens of 1 mol antigen per 2 mol antibody.

Fiber Optic Fluorimeter

The fiber optic fluorimeter is described in detail elsewhere (5,17). Briefly, it consists of a 50 mW argon-ion laser (Omnichrome, Chino, CA), an off-axis parabolic mirror (Melles Griot, Irvine, CA), and a biconvex fused

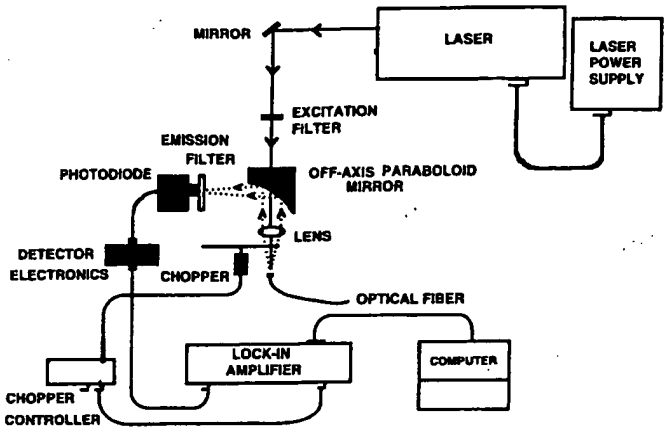

FIG. 1. Fiber optic fluorimeter. This figure represents a schematic of the components of the instrument and the path of the laser light and the returning fluorescent signal.

silica lens (Newport Corp., Fountain Valley, CA). A chopper (Stanford Research Systems, Sunnyvale, CA) is positioned between the focusing lens and the fiber with a photodiode employed for signal conversion. A KV550 filter (Schott Glass Technologies, Duryea, PA) was placed between the photodiode and the parabolic mirror. The chopper and photodiode are interfaced with a lock-in amplifier and data is collected using a lap-top computer (Grid Systems Corp., Fremont, CA).

Figure 1 is a diagram of the fiber optic fluorimeter utilized for this study. The fluorimeter consists of an air-cooled argon-ion laser which generates light at 514 nm. The light beam leaves the laser and passes through an excitation filter to remove plasma lines and any light not of this specific wavelength. The beam passes through the off-axis parabolic mirror and is focused onto the fiber core at the connector end of the fiber. The placement of the chopper between the focusing lens and connector end of the fiber allowed the out of phase fluorescence generated as the excitation light passed through the optical components, as well as stray excitation, to be subtracted from the signal. Since phase sensitive detection narrows the noise band width, a better signal/noise (S/N) ratio is achieved (19).

At the distal end of the fiber, where the cladding has been stripped from the core, an evanescent wave of light extends approximately 100 nm out from the core. The evanescent wave excites the TRITC-labeled antibodies that are bound to the fiber surface as the antibody–antigen–antibody complex forms. The emitted fluorescent light travels back through the fiber core, reflects off the parabolic mirror, and passes through the KV550 filter. This filter blocks any reflected excitation light from the optical components and allows only light greater than 550 nm to be received by the photodiode. The photodiode is interfaced with the chopper through the lock-in amplifier.

Assays

The distal end of each fiber was blocked with an epoxymolybdenum plug to block the light leaving the distal end of the fiber and preventing excitation of bulk fluorescence. All solutions were equilibrated to room temperature before the start of each assay. Fiber probes with immobilized antibodies were incubated in PBS containing 2 mg/ml bovine serum albumin (BSA) for a minimum of 30 min prior to the start of each assay to bleach any fluorescent contaminants of the system. When the baseline drift was less than 1 μV/min, the immunoassays were performed. In the one-step assay, the fiber was inserted into PBS containing 1.0% gelatin, toxin A (50 or 200 ng/ml), and 5 μg/ml TRITC–anti-toxin A IgG. Signal was monitored for a minimum of 10 min. In the two-step assay, the fiber was incubated with toxin A (1, 5, 25, 50, 100, or 200 ng/ml) in PBS with 1.0% gelatin for 10 min. The fiber was removed from this solution and placed in PBS containing 5 μg/ml TRITC–anti-toxin A IgG with 2 mg/ml BSA. Fluorescence signal was monitored for at least 10 min. For each concentration of toxin A tested, a separate fiber was used. Fluorescence signal was measured above the "background" signal. Background included not only the instrument noise but also any fluorescence generated by the TRITC–anti-toxin A IgG in the bulk solution prior to binding of antigen to the fiber surface.

RESULTS

Comparison of Antibodies Used for Immobilization to the Fiber

The fiber optic procedure developed for the detection of botulinum toxin utilizes a sandwich immunoassay that is performed on the surface of a tapered optical fiber. This requires the immobilization of antibodies specific for botulinum toxin onto the surface of the fiber. Once the toxin has become bound at the surface, a second antibody labeled with TRITC is used for signal generation. In the case of all assays, this second antibody is TRITC–anti-toxin A IgG. IgG was isolated from the serum of a horse hyperimmunized with *C. botulinum* type A. A portion of the IgG fraction was affinity purified against toxin A. The IgG fraction and the affinity-purified anti-toxin A IgG were immobilized separately onto optical fibers, and the results compared to those obtained using an immobilized monoclonal antibody, BA11-3.

Figure 2 represents the results obtained for the comparison of the three types of immobilized antibodies. The assay itself is a two-step sandwich immunoassay. The affinity-purified anti-toxin A antibodies were capable of generating higher signal levels than the MAb BA11 or the IgG fraction when compared against the same concentrations of toxin A. The most dramatic dif-

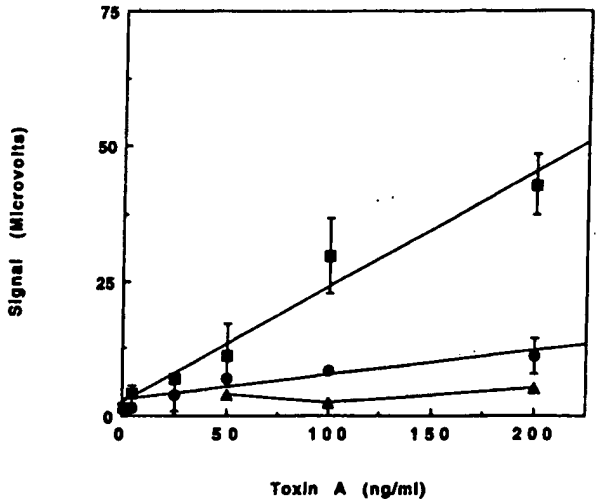

FIG. 2. Comparison of antibodies immobilized. Affinity-purified anti-toxin A antibodies (■), anti-toxin A IgG (●), and monoclonal antibody, BA11-3 (▲), were immobilized to tapered fibers. Fibers were incubated with toxin A for 10 min and placed in TRITC–anti-toxin A IgG (5 μg/ml). Signal levels were recorded at 2 min post incubation with the TRITC solution. Values represent signal minus background and the mean ± SD for three fibers at each concentration.

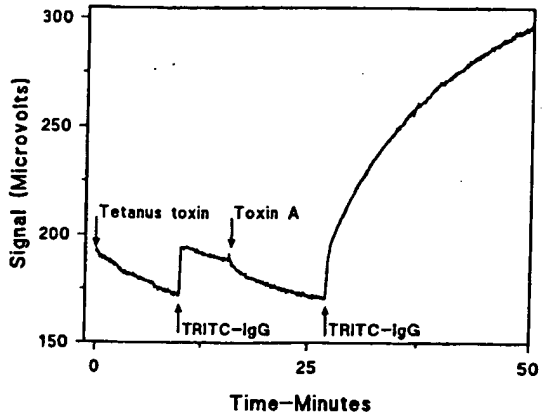

FIG. 3. Specificity of affinity-purified anti-toxin A antibody. Affinity-purified anti-toxin A antibody was immobilized to a tapered optical fiber. The fiber was incubated with tetanus toxin (200 ng/ml) for 10 min, followed by incubation in TRITC–anti-toxin A IgG (5 μg/ml). The same fiber was placed in toxin A (200 ng/ml) for 10 min, followed by incubation in TRITC–anti-toxin A IgG.

ferences were observed at the higher concentrations of toxin A. With assays using 200 ng/ml toxin A, the maximum signal change at 2 min post incubation with TRITC–anti-toxin A IgG was 43 μV using the affinity-purified anti-toxin A antibodies. The maximum change at 2 min post incubation for the IgG fraction immobilized was 13.5 μV and only 5.0 μV using the MAb BA11-3.

Affinity-purified anti-toxin A antibody also detected the lowest concentration of toxin A. At 5 ng/ml toxin A, the signal increased 16.8 μV over background at 10 min post incubation with the TRITC–anti-toxin A IgG. The IgG fraction and the MAb BA11-3 did not produce any change over background. At 25 ng/ml toxin A, the maximum signal increase over background at 10 min post incubation with TRITC–anti-toxin A IgG was 25.8 μV for the affinity-purified anti-toxin A antibody and 11.1 μV for the IgG fraction. The MAb BA11-3 did not produce any significant increase in signal level at this concentration.

Specificity

Affinity-purified anti-toxin A antibody immobilized to the fibers was tested against tetanus toxin as a negative control. The horse had been immunized against tetanus and thus the IgG fraction with which the TRITC–anti-toxin A was prepared probably contains antibodies specific for tetanus toxin. Furthermore, at least one monoclonal antibody against botulinum toxin has been shown to recognize tetanus toxin (20). No response was obtained when the fiber coated with affinity-purified anti-toxin A antibody was incubated for 10 min with 200 ng/ml tetanus toxin and placed in TRITC–anti-toxin A IgG for 10 min. The same fiber was then incubated with 200 ng/ml botulinum toxin A and a signal greater than 75 μV was observed after 10 min incubation in TRITC–anti-toxin A IgG (Fig. 3).

To determine the degree of cross-reactivity between toxin types, affinity-purified anti-toxin B antibodies were immobilized on a fiber and exposed to toxin A and a TRITC-labeled horse IgG known to recognize both A and B toxins. No signal was generated after exposure to the type A toxin, but the same fiber subsequently incubated with type B toxin and the TRITC–IgG responded instantaneously (Fig. 4).

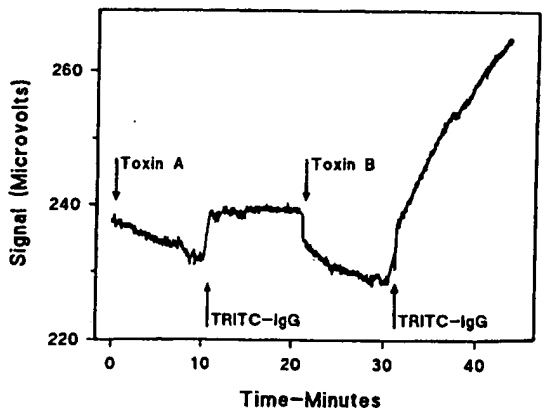

FIG. 4. Specificity between toxin types. Affinity-purified anti-toxin B antibody was immobilized to a step-tapered optical fiber. The fiber was incubated with toxin A (200 ng/ml) for 10 min, followed by a 10-min incubation with TRITC–anti-toxin A,B IgG (5 μg/ml). The same fiber was then incubated in toxin B (200 ng/ml) for 10 min, followed by incubation in TRITC–anti-toxin A,B IgG.

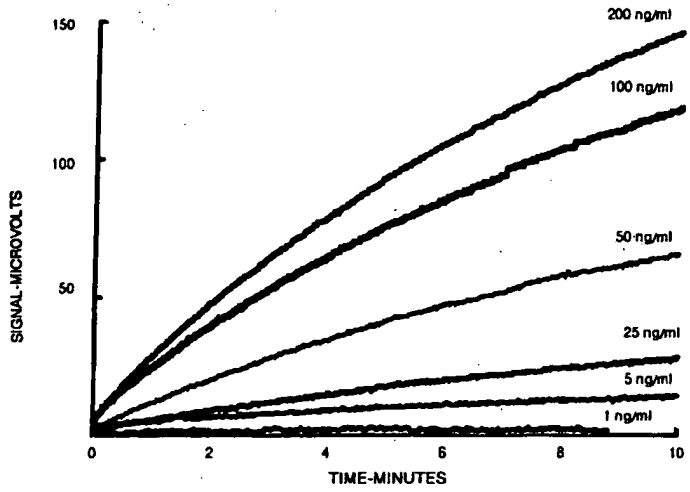

FIG. 5. Sensitivity for *Clostridium botulinum* toxin A. Affinity-purified anti-toxin A antibody was immobilized to tapered optical fibers. For each concentration of botulinum toxin A, a separate fiber was utilized. Each fiber was placed in the toxin A for 10 min before placement in the TRITC–anti-toxin A IgG solution (5 μg/ml). Graph represents a subtraction of background fluorescence upon insertion of the fiber into TRITC–anti-toxin A IgG at time zero. Background levels ranged from 120 to 160 μV. Data represent the results from one of three consistent experiments.

As a control for nonspecific signal generation during the use of fibers coated with monoclonal antibodies, normal mouse IgG was immobilized to fibers and tested against 200 ng/ml toxin A with the same two-step assay. No signal was generated (data not shown).

Sensitivity

Affinity-purified anti-toxin A antibody was immobilized on the fibers for sensitivity testing using the two-step assay. Results presented in Fig. 5 represent a subtraction of the background at the time of placement in the TRITC–anti-toxin A IgG solution. The signal displayed represents the rise in signal over the background fluorescence. Signal levels rise steadily as the time of incubation with the TRITC–anti-toxin A IgG increases. As the concentration of toxin increases, the rate of signal generation and signal magnitude increase. The most pronounced differences in signal change over time occur at the higher concentrations of toxin A. At 1 ng/ml toxin A, the signal generated was only 1–2 μV above the background fluorescence. Each concentration was tested with a separate fiber and data was overlayed onto one graph.

Assay Performance: One Step vs Two Step

A comparison was made between the one-step and two-step procedure using 50 and 200 ng/ml botulinum toxin A. Optical fibers coated with affinity-purified anti-toxin A antibodies were used. In the one-step assay, toxin A and TRITC–anti-toxin A IgG were mixed together in the same solution. The optical fiber was placed into this solution and fluorescence monitored. The two-step procedure was as described previously using a 10-min preincubation in the toxin A solution. Figure 6 represents the results of these two assays using 200 ng/ml toxin A. Background fluorescence from the initial insertion of the fiber into the TRITC–anti-toxin A IgG solution was subtracted and the rise in signal represents the change over the background. Signal strength and rate appear to be very similar for both assays. Similar results were obtained using 50 ng/ml toxin A.

DISCUSSION

The assay relies on the formation of a fluorescent complex between the botulinum toxin, an immobilized capture antibody, and a fluorescent antibody. By utilizing the evanescent wave of the fiber, the binding events along the core of the tapered fiber can be transduced as an increase in fluorescence intensity (21). The continuous measurement permits results to be available within seconds of sample introduction. Fiber optic fluorimeters such as this one are unique, because they can be set up for remote and continuous monitoring. The optical path through the fiber is not subjected to electrical interferences, which could cause problems for other systems.

The fiber optic biosensor requires the immobilization of antibodies to the fiber core for capture of toxin in solution. The results of the sensitivity of assays performed were dependent upon the antibodies used for immobilization to the fiber. Fibers coated with affinity-purified anti-toxin A antibodies were more sensitive and

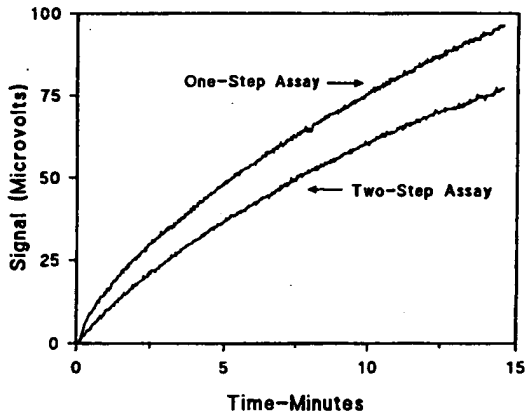

FIG. 6. One-step vs two-step immunoassay. Fibers coated with affinity-purified anti-toxin A antibody were tested against botulinum toxin A. In the one-step assay, the fiber is placed in a solution containing 200 ng/ml toxin A and 5 μg/ml TRITC–anti-toxin A IgG antibody. In the two-step assay, the fiber is incubated with toxin A for 10 min prior to placement in 5 μg/ml TRITC–anti-toxin A IgG.

produced stronger fluorescent signals than the anti-toxin A IgG which was not affinity purified or a monoclonal antibody, BA11-3. The affinity-purified anti-toxin A IgG represented 15% of the total IgG isolated from the horse serum. Our data is consistent with an approximately fivefold more active antibody being immobilized on the fiber surface for the affinity purified compared to the IgG itself. The amount of functional antibody coupled to the surface should have been equivalent for the affinity purified and the MAb BA11-3, thus the difference in signal generated with those antibodies reflects a difference in antibody function rather than surface density. Since the results suggest that the sensitivity of the assay is dependent upon the selection of antibody for immobilization, further research with other monoclonal antibodies is probably worthwhile. The affinity of the antibody for the toxin is an important element of consideration when selecting an antibody. Even though the MAb BA11-3 did not show significant results, it is possible that some of the other monoclonal antibodies against *C. botulinum* toxin which have been reported in the literature (20,22) could help improve the sensitivity of the fiber optic system.

The acute toxicity test in mice presently provides the only assay with sensitivity less than 5 MLD_{50} units/ml, which can be used for the detection of low levels of botulinum toxin in food stuffs and biological samples (20). Using the fiber optic biosensor to detect botulinum toxin, results show sensitivity levels in the low nanogram/milliliter range with analysis in less than a minute. Approximately, 1 ng/ml toxin A is equivalent to 200 MLD_{50} units/ml (11). The fiber optic sensor is currently not as sensitive as the mouse bioassay, but it has considerable potential. Future improvements in the optical components and the tapering angle of the fiber probes, as well as the affinity of antibodies specific for botulinum toxin, may produce two orders of magnitude more sensitivity.

The sensitivity of this fiber optic fluorimeter is comparable in sensitivity, assay time, and S/N ratio to the fiber optic evanescent wave biosensor developed by the Ciba–Corning group (3). The S/N ratio for the concentrations of toxins assayed ranged from 1.2 to 3.0 while Walczak *et al.* report a S/N ratio of about 1 at the lower concentrations of analyte assayed and do not describe the S/N ratio of their system. Walczak *et al.* use B-phycoerythrin as a label which has 32 fluorophores/molecule compared to 2 fluorophores/IgG in our rhodamine-based system. Thus, their report of a 10-fold greater sensitivity in their fluorescence immunoassay indicates equivalent instrument sensitivity. Rogers *et al.* (4) report less sensitivity (nM) and a S/N ratio >99.

It was shown that a one-step assay is sufficient for the rapid detection of botulinum toxin A. Since no prior incubations are necessary, the optical fiber can be placed directly into a sample solution with added signal molecule (TRITC–antibody) for monitoring for the presence of toxin. The fiber will actually collect the toxin as it performs the analysis. The one-step assay would be easy to use in field testing and there would be no potential for cross contamination since the disposable fiber can be replaced after each analysis or after each positive analysis. The assay performed as a one-step test is thus simple, homogeneous, and appropriate for use under field conditions for screening both environmental and clinical samples. The two-step assay would be advantageous when screening a number of different samples. Since the labeled antibody is the reagent used in largest quantity, the two-step procedure could be employed repeatedly utilizing the same solution of TRITC–antibody. The TRITC–antibody solution could be conserved, particularly in situations where the majority of samples are negative.

Another significant advantage of this biosensor-based assay is the specificity for botulinum toxin. When fibers were tested against tetanus toxin, no response was observed even though it is known that these two toxins have some sequence homology (23). Utilizing affinity-purified antibodies against a specific type of toxin seems to impart discrimination between types. When affinity-purified antibodies against toxin B were tested against toxin A, no response was observed. This is ideal when testing for only one type of botulinum toxin.

This fiber optic-based biosensor exhibits a very rapid, real-time analysis for the detection of *C. botulinum* toxin. By careful selection of the immobilized antibody, the assay can be made specific for either type A or type B toxin. As well as specificity, the fiber optic biosensor has the potential of achieving the sensitivity of the methods currently being used for the detection of this very dangerous toxin. This fiber optic assay is a unique approach for the detection of botulinum toxin.

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